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The Spindle Checkpoint Kinase Bub1 and Cyclin E/Cdk2 Both Contribute to the Establishment of Meiotic Metaphase Arrest by Cytostatic Factor

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Summary

In vertebrate unfertilized eggs, metaphase arrest in Meiosis II is mediated by an activity known as cytostatic factor (CSF) [1]. CSF arrest is dependent upon Mos-dependent activation of the MAPK/Rsk pathway [2–6], and Rsk activates the spindle checkpoint kinase Bub1, leading to inhibition of the anaphase-promoting complex (APC), an E3 ubiquitin ligase required for the metaphase/anaphase transition [7–9]. However, it is not known whether Bub1 is required for the establishment of CSF arrest or whether other pathways also contribute. Here, we show that immunodepletion of Bub1 from egg extracts blocks the ability of Mos to establish CSF arrest, and arrest can be restored by the addition of wild-type, but not kinase-dead, Bub1. The appearance of CSF arrest at Meiosis II may result from coexpression of cyclin E/Cdk2 with the MAPK/Bub1 pathway. Cyclin E/Cdk2 was able to cause metaphase arrest in egg extracts even in the absence of Mos and could also inhibit cyclin B degradation in oocytes when expressed at anaphase of Meiosis I. Once it has been established, metaphase arrest can be maintained in the absence of MAPK, Bub1, or cyclin E/Cdk2 activity. Both pathways are independent of each other, but each appears to block activation of the APC, which is required for cyclin B degradation and the metaphase/anaphase transition.

Results and Discussion

Classically, cytostatic factor (CSF) activity is bioassayed by microinjection into one blastomere of a 2-cell embryo; the injected blastomere arrests cleavage at the next mitosis with an intact metaphase spindle, a high level of cyclin B, and high cyclin B/Cdc2 activity [1]. However, CSF arrest at metaphase can also be induced by the addition of Mos to extracts prepared from eggs activated for 30 or 40 min with the calcium ionophore A23187 [6, 10]. Such extracts lack endogenous Mos and normally cycle in vitro through up to three rounds of DNA replication and mitosis with corresponding activation and inactivation of cyclin B/Cdc2 histone H1 kinase activity through synthesis and degradation of cyclin B [11]. However, in the presence of Mos, extracts arrest in M phase with elevated cyclin B/Cdc2 activity and condensed chromosomes [6, 10]. An advantage of the egg extract assay is that it facilitates depletion/reconstitution approaches to the study of CSF. Using this assay, Bhatt and Ferrell [6] showed that depletion of the MAPK substrate $p90^{Rsk}$ blocks establishment of CSF arrest by Mos, and arrest can be restored by the addition of recombinant $p90^{Rsk}$. This indicates that $p90^{Rsk}$ is necessary for CSF arrest.

Studies in maturing oocytes inhibiting Mos or using the MAPKK inhibitor UO126 showed that the MAPK pathway partially inhibits the anaphase-promoting complex (APC) and suppresses S phase entry [12–14]. APC activation resulting from loss of MAPK activity could be prevented by constitutively active Rsk even in the presence of U0126 [12]. The APC is also inhibited in a MAPK-dependent manner in the nocodazole-induced spindle assembly checkpoint in mammalian cells [15–17], and the pathway of spindle checkpoint regulation by the Bub and Mad proteins is thought to be conserved in higher eukaryotes [9]. The possibility that MAPK/Rsk might effect CSF arrest through the spindle checkpoint pathway was suggested by the finding that the spindle checkpoint kinase Bub1 is activated by $p90^{Rsk}$ through direct phosphorylation [7]. However, the spindle checkpoint is usually defined as nocodazole dependent, but CSF arrest occurs with an intact spindle and is unaffected by nocodazole. To directly evaluate a role for Bub1 in CSF arrest, we used depletion/reconstitution approaches in egg extracts. Figure 1A shows the histone H1 kinase activity of a control extract cycling at 22°C after the addition of sperm nuclei. M phase occurred after 15 min of incubation, as judged both by a peak of histone H1 kinase activity 4- to 5-fold above the interphase level and by condensed chromosomes (Figure 1F). By 30–45 min of incubation, histone H1 kinase had declined to less than 2-fold above the initial level, and nuclei were in interphase. However, if recombinant GST-Mos was added at time 0 to the extract, M phase persisted for at least 1 hr beyond 15 min, as judged by nuclear morphology in which chromosomes are highly condensed (Figure 1F) and by histone H1 kinase activity greater than 2-fold above the interphase level (Figure 1B). This represents a stable Mos-dependent CSF arrest in vitro, as described previously [6]. Interphase extracts completely depleted of Bub1 (Figure 1G) were unable to establish CSF arrest after the addition of Mos (Figures 1C and 1F). To exclude the possibility that a Bub-associated component was responsible for loss of CSF arrest, recombinant Bub1 was added back to immunodepleted extracts at the time of Mos addition. As shown in Figures 1D, 1E, and 1F, wild-type, but not kinase-dead, Bub1 was able to restore Mos-dependent CSF arrest to depleted extracts, as judged both by histone H1 kinase activity and the formation of condensed chromosomes. These results establish that the kinase activity of Bub1 is required for the establishment of CSF arrest. They support a model in which the Mos/MAPK/Rsk-dependent activation of Bub1 activates the spindle checkpoint pathway to inhibit the APC and prevent the metaphase/anaphase transition during CSF arrest [7].

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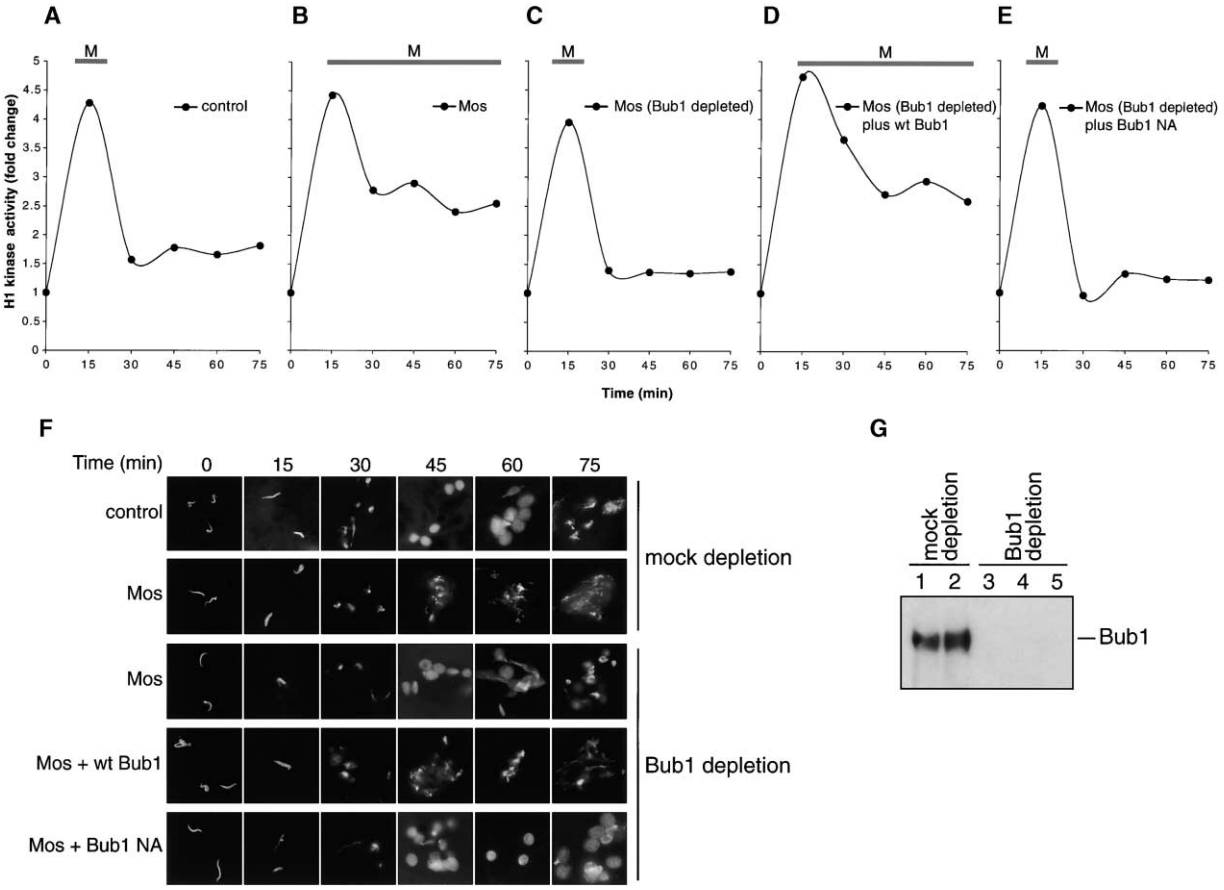

Figure 1. The Kinase Activity of Bub1 Is Required to Establish CSF Arrest

(A–E) Histone H1 kinase activity. Extracts in interphase at 4°C were treated with Dynabeads with or without bound anti-Bub1 antibodies to immunodeplete Bub1 (Figure 1G) and were then supplemented with sperm nuclei, Mos, and wild-type or kinase-dead Bub1 as indicated. Cycling was initiated by warming to 22°C, and aliquots of the extracts were monitored for histone H1 kinase activity as described in the Experimental Procedures. The "M" bar in each panel refers to the length of M phase as determined by DAPI staining (Figure 1F). Histone H1 kinase activity, which is more than 2-fold above the initial (interphase) level, was consistently associated with M phase, whereas extracts with activity less than 2-fold above the initial level were consistently in interphase.

(F) Effect of Bub1 depletion on nuclear morphology in cycling egg extracts. The samples shown in (A)–(E) were also monitored for progression of the cell cycle by DAPI staining and fluorescence microscopy at the indicated times. Nuclei in all samples entered mitosis by 30 min of incubation, and samples treated with control beads progressed into the next interphase by 45 min. Addition of Mos at time 0 caused a prolonged M phase in the presence, but not the absence, of Bub1. Arrest could be restored by the addition of wild-type (wt) Bub1, but not kinase dead (NA) Bub1.

(G) Immunodepletion of Bub1. Rabbit polyclonal antibody to Bub1 was bound to Protein A Dynabeads and incubated with a cycling extract in interphase at 4°C for 30 min. Controls were incubated with beads bound with control IgG. The supernatants were collected and immunoblotted for the presence of Bub1.

An important question concerns why CSF arrest occurs only in Meiosis II and not in Meiosis I. All of the known elements required to generate CSF activity are present and active at metaphase of Meiosis I, including Mos, MAPK, $p90^{Rsk}$, and Bub1, and these are essential for suppression of S phase between the meiotic divisions [2, 12–14]. One possible explanation is that another pathway appearing in Meiosis II also contributes to CSF arrest. Cyclin E and Cdk2 are synthesized de novo during Meiosis II, and the kinase-active complex assembled during meiosis persists in the early embryo until the midblastula transition [18, 19]. Antisense oligonucleotides against Cdk2, when injected into resting G2 phase oocytes, have been reported to cause failure of CSF arrest [20], whereas injection of the Cdk2 inhibitor $p21^{Cip1}$ has been reported not to prevent CSF arrest at Meiosis II [21]. In recent times, it has become evident that this question should be reinvestigated with gain-of-function reagents rather than loss-of-function reagents, because relatively little cyclin E/Cdk2 activity might be sufficient for contributing to CSF activity, especially if it is localized at a specific site such as the kinetochore. This situation appears to be the case with active MAPK on kinetochores in mammalian cells, which constitutes less than 1% of total MAPK in the cell but is necessary for spindle checkpoint function [15–17].

Inasmuch as cyclin E/Cdk2 is inactivated by Tyr-15 phosphorylation upon CSF release and periodically during *Xenopus* embryonic cell cycles [18, 22, 23], we expressed in Sf9 cells a cyclin E/Cdk2 complex in which

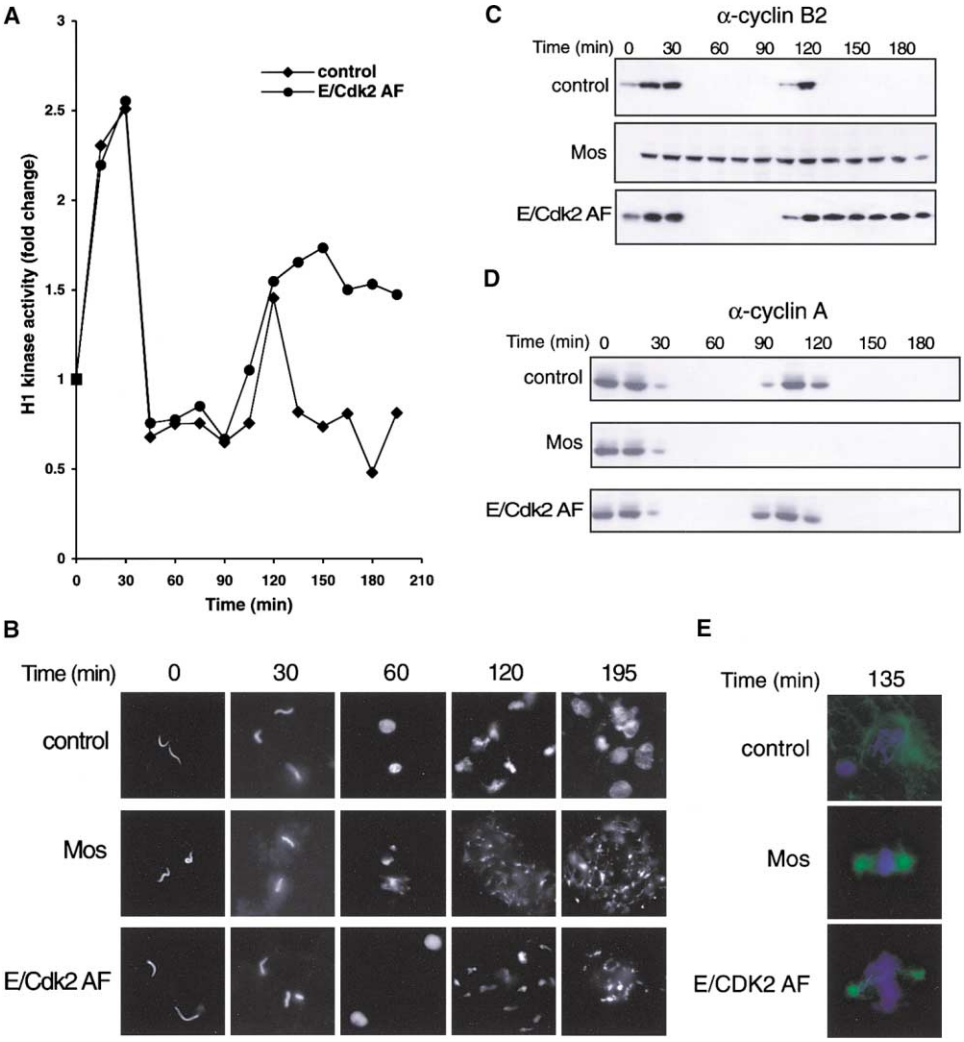

Figure 2. Cyclin E/Cdk2 Has CSF Activity in Egg Extracts

(A) Cyclin E/Cdk2 AF maintains mitotic histone H1 kinase activity in cycling egg extracts. Cycling extracts were prepared as described in the Experimental Procedures, and cycling was initiated at time 0 by warming to 22°C in the presence of the indicated agents. Cyclin E/Cdk2 AF (200 nM) caused arrest in the second cycle. The histone H1 kinase activity of the added cyclin E/Cdk2 was less than 1% of the total histone H1 kinase activity.

(B) Chromosome condensation in cycling egg extracts. Aliquots of the extracts described in (A) were supplemented with DAPI and then examined by fluorescence microscopy.

(C) Immunoblotting of Cyclin B2. At the indicated times, samples of the extracts in (A), and also one containing GST-MOS, as indicated on the left, were immunoblotted for the level of cyclin B2 after initiation of cycling by warming to 22°C.

(D) Immunoblotting of cyclin A. The samples in (C) were immunoblotted for the level of cyclin A1.

(E) Formation of metaphase spindles with cyclin E/Cdk2 AF. From an extract like that shown in (A), supplemented with the indicated components, spindles then isolated on coverslips 135 min after initiation of cycling and then stained for α-tubulin (green) and DNA (blue) as described in the Experimental Procedures.

Thr-14 and Tyr-15 of Cdk2 were mutated to alanine and phenylalanine, respectively. As shown in Figure 2A, cyclin E/Cdk2 AF added to an interphase extract was able to cause mitotic arrest in the second cycle, as judged by histone H1 kinase activity, condensed chromosomes, and stabilization of cyclin B. This occurred in the absence of detectable MAPK activity or Mos (data not shown). The M phase arrest appeared to be at metaphase, as judged by maintenance of maximal histone H1 kinase activity, maximum cyclin B, low cyclin A, condensed chromosome morphology, and spindles arrested at metaphase (Figure 2). These results support the suggestion that cyclin E/Cdk2 might contribute to CSF arrest [20]. They are also consistent with recent evidence that Cdk2 inactivation by a PKA-dependent pathway is necessary for cyclin B degradation [23].

Since CSF arrest is associated with inhibition of APC activity, we predict that the expression of cyclin E/Cdk2 in Meiosis I should produce inhibitory effects on the APC at anaphase I. To investigate this possibility directly, cyclin E/GST-Cdk2 AF was injected into oocytes just after germinal vesicle breakdown (GVBD) and shortly before metaphase I. Controls were injected with GST. As shown in Figure 3, total histone H1 kinase activ-

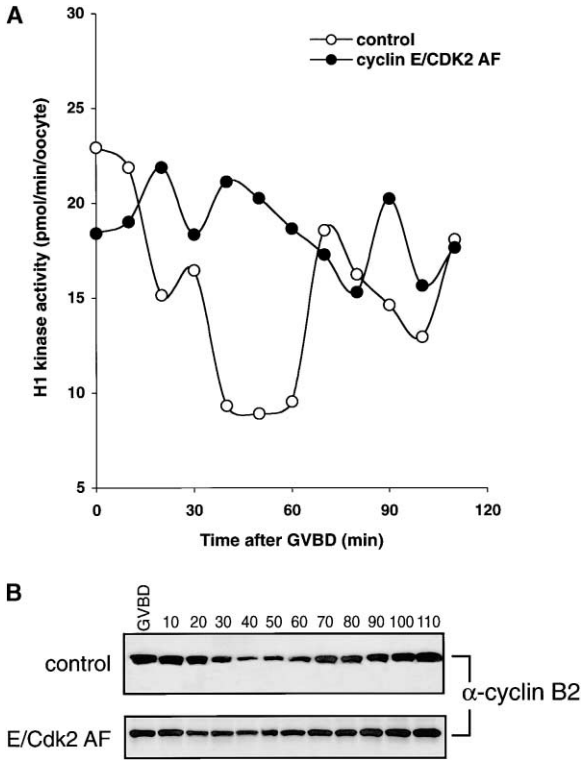

Figure 3. Effect of Cyclin E/Cdk2 AF in Meiosis I

(A) Histone H1 kinase activity at anaphase I. Progesterone-treated oocytes were microinjected shortly after GVBD with 50 nM cyclin E/GST-Cdk2 AF or GST alone (control) as described in the Experimental Procedures. At the indicated times, oocytes were homogenized, and clarified extracts were assayed for histone H1 kinase activity. The units of H1 kinase activity accounted for by the cyclin E/Cdk2 AF itself were less than 1% of the initial histone H1 kinase activity in the extract.

(B) The level of cyclin B2 at anaphase I. Aliquots of the samples in (A) were immunoblotted at the indicated times for the level of cyclin B2 as described in the Experimental Procedures.

ity in controls decreased transiently to approximately 50% of the metaphase I level during the Meiosis I/II transition, and approximately 50% of cyclin B2 was degraded, as reported previously [12, 24, 25]. In the presence of cyclin E/Cdk2 AF, histone H1 kinase activity was maintained at a much higher level, near the maximal metaphase I level (Figure 3A), and cyclin B2 degradation was almost completely inhibited (Figure 3B). These results support the concept that cyclin E/Cdk2 inhibits APC-mediated cyclin B degradation and may contribute to the establishment of CSF arrest.

While this work was in progress, Sharp-Baker and Chen [26] reported that depletion of Bub1 from a CSF extract did not cause exit from metaphase arrest but was required for establishing the spindle assembly checkpoint in the presence of a large number of nuclei and nocodazole. Other work established that $p90^{Rsk}$ is required to establish Mos-dependent CSF arrest in egg extracts, but is not required to maintain metaphase arrest once it has been established [6]. These results raise the possibility that Bub1 is also required only to establish, but not to maintain, CSF arrest. To address this possibility, Bub1 was depleted from a CSF-arrested, unfertilized egg extract, and maintenance of metaphase arrest was monitored by histone H1 kinase activity. Depletion of Bub1 did not cause exit from CSF arrest in the unfertilized egg extract (Figure 4A), although it is required to establish CSF arrest (Figure 1). Bub1 activation is a direct consequence of MAPK/Rsk activity, and inhibition of MAPK/Rsk/Bub1 activation by U0126 blocks the establishment of CSF arrest in oocytes [7, 12]. Therefore, it can be predicted that MAPK is also not required, and it is also of interest to evaluate whether cyclin E/Cdk2 is required to maintain CSF arrest. CSF-arrested egg extracts were treated with U0126 or $p27^{Xic1}$, an inhibitor of cyclin E/Cdk2, and maintenance of M phase was monitored. As shown in Figure 4B, neither U0126 nor $p27^{Xic1}$, which fully inhibited MAPK activity and cyclin E/Cdk2 activity, respectively (Figures 4C and 4D), caused loss of CSF activity when administered alone or in combination (Figure 4B). These results are consistent with evidence that $p21^{Cip1}$ injection into CSF-arrested oocytes also does not cause CSF release [21].

One explanation for why MAPK, Rsk, and Bub1 may not be required to maintain CSF arrest is that multiple feedback loops exist in M phase, and these may provide redundant pathways to maintain APC inhibition in M phase. A precedent for this idea has come from studies of the activation of the polo-like kinase Plx1. In some systems [27], but not others [28], Plx1 activation is dependent on basal cyclin B/Cdc2 activity at the G_2/M transition; however, once M phase has been established, complete inhibition of cyclin B/Cdc2 by $p21^{Cip1}$ has no effect on the activation state of Cdc25C or Plx1 [28, 29], presumably because of feedback loops that maintain these activities even in the absence of cyclin B/Cdc2 activity.

The ability of Bub1 (Figure 1), Mad2p [30], and Fizzy/Cdc20 [30–32] to regulate CSF arrest suggests that Bub1 promotes meiotic metaphase arrest by the same mechanism used in mitotic cell spindle checkpoint arrest. The MAPK/Rsk linkage seen with CSF arrest is likely to be similar to that present on kinetochores in mammalian cells before anaphase [15–17]. In mammalian cells, active MAPK during both normal mitosis and after spindle checkpoint activation by nocodazole is confined to kinetochores and spindle poles [15, 16], but the Mos-dependent global upregulation of the MAPK pathway in oocytes may activate the spindle checkpoint pathway for CSF arrest independently of microtubule attachment to kinetochores [7].

The results reported here establish cyclin E/Cdk2 as another element appearing in Meiosis II that may contribute to the inhibition of the APC during CSF arrest. Cyclin E/Cdk2 AF was able to inhibit the APC to maintain a metaphase level of cyclin B both when expressed in Meiosis I and when expressed in egg extracts. These effects appear to be independent of Mos, inasmuch as cyclin E/Cdk2 AF produces metaphase arrest in the absence of Mos (Figure 2), and, conversely, Mos can produce arrest in the absence of endogenous cyclin E/Cdk2 activity (data not shown). These results extend the work of Grieco and colleagues, who suggest that changes in PKA activity in the extract regulate Tyr-15 phosphorylation of Cdk2 and exit from mitosis [23]. However, extracts arrested in M phase by low PKA activity have high levels of both cyclin A and cyclin B [23],

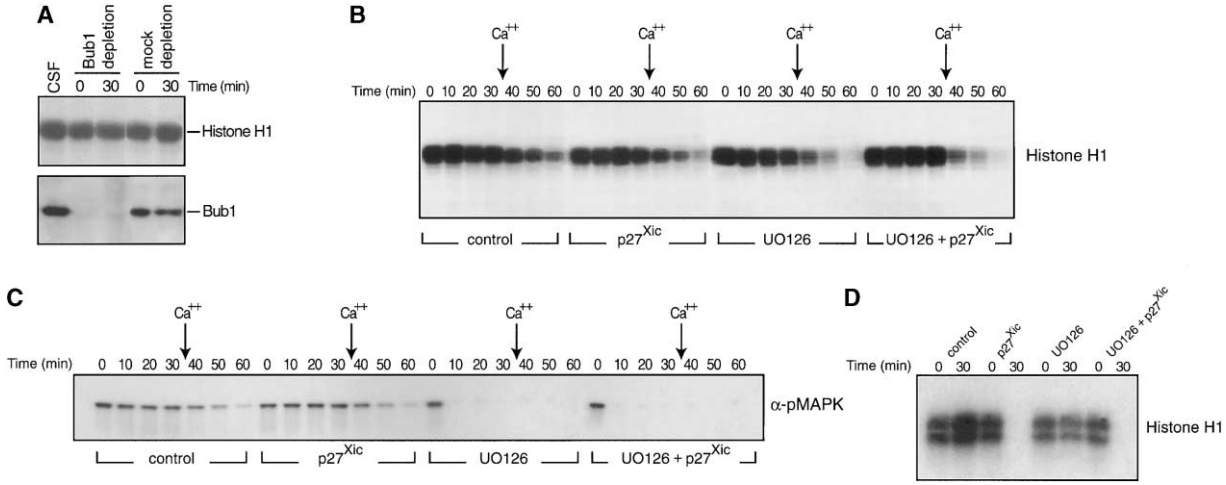

Figure 4. Bub1, MAPK, and Cyclin E/Cdk2 Are Not Required to Maintain CSF Arrest

(A) Bub1 depletion. The histone H1 kinase activity of a CSF-arrested extract from unfertilized eggs was monitored for 30 min with or without prior quantitative immunodepletion of Bub1 from the extract. The upper panel shows an autoradiograph of radiolabeled histone H1, and the lower panel is an immunoblot of Bub1.

(B) The MAPK and cyclin E/Cdk2 pathways are not required to maintain CSF arrest. CSF-arrested extracts from unfertilized eggs were incubated for 30 min at 22°C. Then, Ca^{2+} was added to cause CSF release, and the histone H1 kinase activity was monitored for 30 additional min in control extracts, in extracts supplemented with 50 μM U0126 or 20 nM $p27^{Xic}$, or both, as indicated. CSF arrest was maintained with all treatments, and CSF release with calcium occurred with the same kinetics in each case.

(C) MAPK activity. The extracts described in (B) were immunoblotted with an anti-active phospho-MAPK antibody.

(D) Inhibition of cyclin E/Cdk2 by $p27^{Xic1}$. CSF-arrested extracts from unfertilized eggs were supplemented with 20 nM $p27^{Xic1}$, 50 μM U0126, or both, as indicated. Cyclin E/Cdk2 was immunoprecipitated from the extracts, and the histone H1 kinase activity of the immunoprecipitate was determined initially and after 30 min. An autoradiograph is shown.

indicating that arrest occurs at a point earlier than with CSF arrest or spindle checkpoint activation, which stabilize only cyclin B ([33, 34] and Figure 2).

The mechanism by which cyclin E/Cdk2 AF inhibits the APC may relate to the involvement of G_1 cyclin/Cdk complexes in APC inactivation late in G1 or at the onset of S phase [35, 36]. In yeast, APC inactivation in G_1 has been proposed to occur via phosphorylation of the Hct1/Cdh1/Fizzy-related protein by G_1 cyclin/Cdks, resulting in loss of APC activity toward mitotic cyclins [37]. Such a mechanism is unlikely to explain the actions of cyclin E/Cdk2 AF in *Xenopus*, however, because the *Xenopus* homolog of Hct1/Cdh1 is not expressed until after the midblastula transition in development [30].

Another APC inhibitor, Emi1, has been reported to be present at a low level in unfertilized eggs and in early embryos [38, 39]. It has been suggested that Emi1 acts early in mitosis by binding Fizzy/Cdc20 to prevent premature cyclin B degradation following cyclin B/Cdc2 activation [38]. Emi1 overexpression can block CSF release by calcium, presumably by sequestering Fizzy/Cdc20, and depletion of Emi1 from CSF-arrested extracts causes CSF release, most likely due to the release of Cdc20/Fizzy from Emi1 observed during immunodepletion [40]. However, Emi1 is synthesized and degraded in each mitotic embryonic cycle and increases after fertilization, whereas CSF activity disappears. Emi1 expression also stabilizes cyclin A [38], indicative of arrest at prometaphase, whereas CSF arrest by either Mos or cyclin E/Cdk2 AF and the spindle checkpoint does not prevent cyclin A degradation and exhibits arrest at metaphase (Figure 2 and [33, 34, 41, 42]). These results suggest that mitotic arrest by Emi1 is distinct from CSF arrest. Recently, it was reported that human separase is inhibited in metaphase by phosphorylation at a single Cdk consensus site [43]. Whether direct phosphorylation and inhibition of *Xenopus* separase occurs with cyclin E/Cdk2 merits further investigation.

Conclusions

CSF activity in Meiosis II in vertebrates may be a consequence of the unique presence in Meiosis II of both an active MAPK-dependent spindle checkpoint pathway and an active G_1 cyclin/Cdk complex (Figure 5). The

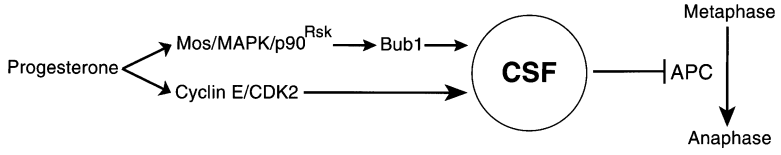

Figure 5. Model For the Generation of CSF Activity

The model indicates that inhibition of the APC by CSF results from the combined action of two independent pathways that are uniquely present together in Meiosis II. One is the Mos-dependent pathway, which upregulates the Bub1-dependent spindle assembly checkpoint, and the other is the cyclin E/Cdk2 pathway, which may inhibit the APC by a pathway normally found in G_1 phase of the cell cycle.

convergence of two pathways for APC inhibition may be important for ensuring the stability of the metaphase II arrest by CSF, which is crucial for completion of meiosis, normal fertilization, and zygotic development. Previously, it was shown that the linkage of the consecutive M phases in Meiosis I and II requires the Mos/MAPK/Rsk pathway and results in only partial APC-mediated cyclin B degradation at anaphase I [12–14, 24, 25]. Our data show that APC inhibition during CSF arrest is likely even greater due to the appearance of cyclin E/Cdk2, since expression of cyclin E/Cdk2 AF at anaphase I (Figure 3) caused greater APC inhibition than was evident in controls. Both pathways are known to be inactivated after CSF release by calcium at fertilization: Mos by proteolysis [2], and cyclin E/Cdk2 by Tyr-15 phosphorylation [23].

Experimental Procedures

Antibodies against pMAPK, cyclin B2, Bub1, cyclin E, and Mos and immunological procedures have been described previously [5, 7, 15, 16, 25]. Immunodepletion of Bub1 was performed essentially as described by Sharp-Baker and Chen [26]. Recombinant 6xHis-tagged wild-type and kinase-dead Bub1 (Bub1 N191A; Bub1 NA) were expressed in Sf9 cells and were purified on Talon beads as described previously [7]. GST, GST-p27^{Xic1}, GST-Cdk2, GST-Cdk2AF, and cyclin E expressed in Sf9 cells were purified on glutathione-agarose beads as described previously [22].

Oocytes were induced to mature with progesterone (1 μg/ml) as described previously [12]. For injection experiments, oocytes were collected within 10 min of GVBD, as monitored by a well-defined white spot on the animal pole. Such oocytes generally underwent anaphase I within 30–60 min. Cycling egg extracts were prepared in interphase as described by Murray [11], except that eggs were crushed 45 min after treatment with the calcium ionophore A23187. This timing proved necessary to allow complete degradation of endogenous Mos. Cycling was initiated by the addition of demembranated sperm nuclei (500/μl) and warming to 22°C. For immunofluorescence, 1-μl aliquots were mixed with 2 μl DAPI (1 μg/ml) and then examined by fluorescence microscopy using an Olympus BH-2 microscope. Spindles were isolated by the method of Desai et al. [44]. Microtubules were stained with a monoclonal antibody to α-tubulin (Sigma) and DNA with Sytox green as described [12, 32].

Acknowledgments

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